

Appendix B

***Clean Version of the Specification
Application No: 09/388,090***

At page 3, line 9

One object of this invention is to identify and provide a novel and highly conserved protein (referred to hereafter and in the claims as "NGSP") from *Neisseria spp.*, preferably *Neisseria gonorrhoeae*, *Neisseria ovis*, *Neisseria lacunata*, *Neisseria osloensis*, and *Neisseria bovis*. The protein of the present invention has a molecular weight of approximately 40-55 kD, and has limited similarity (~36% identity overall) to the DegP (HtrA) protein of *E. coli* (% identity determined using TBLASTP program (Altschul et al., 1990, J. Molec. Biol. 215:403-10; Altschul et al., 1997, Nuc. Acids Res. 25:3389-3402) with data entered using FASTA format; expect 10 filter default; description 100, alignment) and has not been previously identified in any *Neisseria spp.* The protein sequence which is another object of this invention has similarity to several DegP/HtrA-like serine proteases from two other bacteria and these sequence homologies have not been previously reported for any *Neisseria spp.*

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The present invention encompasses the NGSP polypeptide of *Neisseria gonorrhoeae* and other *Neisseria spp.*, including but not limited to, *Neisseria ovis*, *Neisseria lacunata*, *Neisseria osloensis*, and *Neisseria bovis*, having a molecular weight, as determined from the deduced amino acid sequence, of 40 kD to about 55 kD, in isolated or recombinant form. A homologous protein, NMA SP, from *Neisseria meningitidis*, is described and claimed in copending application of Applicants entitled "*Neisseria meningitidis* Polypeptide, Gene Sequence And Uses Thereof"(Application No.: 09/388,089) filed on even date herewith, which is hereby incorporated by reference in its entirety. The present invention encompasses a purified NGSP polypeptide, polypeptides including fragments, derived therefrom (NGSP-derived polypeptides), and methods for making said polypeptide and derived polypeptides. The invention also encompasses antisera and antibodies, including cytotoxic or bactericidal

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antibodies, which bind to and are specific for the NGSP polypeptide, NGSP-derived polypeptides and/or fragments thereof.

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The *E. coli* DegP (HtrA) amino acid sequence available from GeneBank was employed as a BLAST (TBLASTN) subject query to search the genomic sequence databases for *N. gonorrhoeae* (Univ. Oklahoma, USA) strain 1090 to identify linear amino acid sequences that might share some similarity to the DegP protein. No predicted amino acid sequences from this *Neisseria* database showed more than ~30-35% similarity to the *E. coli* DegP protein sequence. Candidate *N. gonorrhoeae* NGSP amino acid sequences were localized within specific genomic DNA sequence “contigs”, and putative open reading frames encoding these NGSP sequences were derived. Putative ORFs capable of encoding proteins of ~40-55 kD, the average size of most DegP-like serine proteases, were then selected and further analyzed for the presence and appropriate relative spacing of semi-conserved catalytic residues (H, D, S) thought to be required for serine protease activity. A single putative open reading frame from the *N. gonorrhoeae* database was identified which met these criteria.

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N. gonorrhoeae strain GC340 was obtained from the Centers for Disease Control and Prevention (CDC). GC340 was streaked on gonococcal agar base (GC agar™, Difco) containing 1.0% IsoVitale™ X (BBL) and grown at 35-37°C in 5% CO₂ for ~24-28 hours. To prepare confluent “lawns” of cells for DNA isolation, three or four single colonies were picked from the “overnight” seed plate and used to inoculate fresh GC plates which were again grown overnight at 35-37°C in 5% CO₂. Cells were collected from the surface of the agar plates by gentle rinsing using trypticase soy broth (TSB) containing 10% glycerol and then stored at -20°C. When needed, cells were thawed at room temperature and bacteria collected by centrifugation in a Sorval SS34 rotor at ~2000 Xg for 15 minutes at room temperature. The supernatant was removed and the cell pellet suspended in ~5.0ml of sterile

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water. An equal volume of lysis buffer (200mM NaCl, 20mM EDTA, 40mM Tris-HCl pH8.0, 0.5% (w/v) SDS, 0.5% (v/v) 2-mercaptoethanol, and 250ug/ml of proteinase K) was added and the cells suspended by gentle agitation and trituration. The cell suspension was then incubated ~12hours at 50°C to lyse the bacteria and liberate chromosomal DNA. Proteinaceous material was precipitated by the addition of 5.0ml of saturated NaCl (~6.0M, in sterile water) and centrifugation at ~5,500 X g in a Sorval SS34 rotor at room temperature. Chromosomal DNA was precipitated from the cleared supernatant by the addition of two volumes of 100% ethanol. Aggregated DNA was collected and washed using gentle agitation in a small volume of a 70% ethanol solution. Purified chromosomal DNA was suspended in sterile water and allowed to dissolve/disburse overnight at 4°C by gentle rocking. The concentration of dissolved DNA was determined spectrophotometrically at 260nm using an extinction coefficient of 1.0 O.D. unit ~50ug/ml.

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A general process for the purification of NGSP protein as a soluble protein is given below. Insoluble material is removed after French press disruption by high speed centrifugation (~10,000Xg, 4°C, 30min). The soluble fraction containing NGSP is suspended in ~20ml of ice cold 50mM Tris-HCl buffer (pH8.0) and loaded onto a DEAE-SEPHACEL™ (Diethylaminoethyl cellulose) (Pharmacia) ionic exchange column (~5cm X 60cm). To minimize autoproteolysis of the NGSP protein, chromatography is conducted at 4°C. Unbound material is washed from the column using loading buffer (50mM Tris-HCl, pH8.0) prior to elution of bound NGSP protein. Elution of NGSP from the SEPHACEL™ (cellulose) matrix is achieved using a NaCl gradient (0.05 - 0.5M NaCl, in 50mM Tris-HCl, pH8.0). Fractions released by the salt gradient are collected and examined by standard SDS-gel electrophoresis methodologies for the presence of a ~40-55 kd protein. Fractions are also assayed for protease activity using a standard azocasein colorimetric assay. Fractions containing NGSP are pooled and extensively dialyzed against 10mM sodium phosphate buffer (SPB, pH8.0) at 4°C.

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The sequence information shown above is used to design a pair of nondegenerate convergent (i.e., one forward and one reverse primer) oligonucleotide primers. PCR amplification of DNA fragments is performed under the same conditions as described above with the exception that the annealing temperature is raised to 50°C. The DNA fragment is isolated from an agarose gel as before and radiolabelled using (32P)-gamma-ATP and T4 polynucleotide kinase according to standard methods. Unincorporated radiolabel is separated from the probe on a G25 SEPHAROSE™ (Agarose) spin column. Before use, the probe is denatured for 2 min. at 95°C and subsequently chilled on ice (4°C).

Appendix D

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Application No: 09/388,090
Docket No.: 7969-082-999

37. (Four Times Amended) An isolated nucleic acid comprising a nucleotide sequence encoding an isolated non-cytosolic polypeptide of a *Neisseria* spp. (NGSP) polypeptide, which is a polypeptide of a *Neisseria* species with the proviso that said *Neisseria* species is not *N. meningitidis*, which polypeptide has a molecular weight of about 40 kD to about 55 kD as determined in SDS polyacrylamide gel electrophoresis using glutamic dehydrogenase, carbonic anhydrase and myoglobin-blue as molecular weight markers, and which polypeptide specifically binds an antibody that specifically binds to a polypeptide encoded by the nucleotide sequence of SEQ ID NO. 3.

38. (Four Times Amended) An isolated nucleic acid comprising the nucleotide sequence of SEQ ID NO. 3.

39. (Thrice Amended) An isolated nucleic acid encoding a NGSP *Neisseria* sp. polypeptide which comprises a nucleotide sequence that hybridizes at 68°C in 0.5M NaHPO₄ (pH 7.2) 1 mM ethylenediaminetetra acetic acid (EDTA)/7% SDS or at 65°C in 6X sodium chloride/sodium citrate (SSC), 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% polyvinyl pyrrol PVP, 0.02% Ficoll, 0.02% BSA or in 50% formamide/0.25 M NaHPO₄ (pH 7.2)/0.25 M NaCl/1 mM EDTA/7% SDS to the sequence of nucleotide SEQ ID NO. 3 or the complement thereof, wherein said complement is complementary to at least 25 contiguous nucleotides of SEQ ID NO. 3.

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48. An isolated nucleic acid encoding a fragment of a NGSP polypeptide said fragment comprising at least 7 amino acids and having an antigenic epitope of the amino acid sequence of the polypeptide encoded by SEQ ID NO. 3.

49. An isolated nucleic acid encoding a NGSP polypeptide which specifically binds an antibody that specifically binds to a polypeptide encoded by SEQ ID NO. 3.

50. (Once Amended) A pharmaceutical composition comprising the isolated nucleic acid of any one of claims 37, 38, 39, 48 or 49.

51. A recombinant expression vector comprising the isolated nucleic acid of any one of claims 37, 38, 39, 48 or 49.

52. A host cell transformed with the recombinant vector of claim 51.

56. (New) An isolated nucleic acid comprising a nucleotide sequence according a polypeptide which comprises the amino acid sequence of SEQ ID NO.: 4.